Apoptosis in the Endothelium of Human Corneas for Transplantation

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PURPOSE. To determine whether endothelial cell loss of human corneas stored in organ culture before transplantation is due to apoptosis.

METHODS. The corneal endothelium of human corneas, stored in organ culture at 34°C for varying periods of time, were analyzed for the presence of apoptotic cells using the TdT-mediated dUTP nick-end labeling (TUNEL) technique. Corneal endothelial cell apoptosis was confirmed by Hoechst staining and immunolabeling with anti-caspase-3 active antibody.

RESULTS. Apoptotic cells were identified in the corneal endothelium of human organ cultured corneas: their number and distribution demonstrated a close correlation with corneal folding and overall quality of the corneal endothelium. TUNEL-positive labeling of cells was confirmed as apoptotic by the presence of morphologic nuclear alterations identified by Hoechst staining and the presence of immunostaining for caspase-3 activity. Corneal endothelial cell apoptosis was independent of cause of donor death, death to enucleation time, and death to culture times.

CONCLUSIONS. Corneal endothelial cell apoptosis appears to determine the suitability of a cornea for transplantation. (Invest Ophthalmol Vis Sci. 2000;41:2887–2893)

The UK Corneal Transplant Service (CTS) Eye Banks provide organ-cultured donor corneas for over 2500 corneal transplants per annum. The success of corneal transplantation is dependent on a healthy endothelium in the donor cornea,1 which is essential to maintain corneal transparency.2

The nonregenerative capacity of human corneal endothelial cells (CECs) necessitates the requirement of a storage technique that will maintain the metabolic function and integrity of donor cadaver corneal endothelium until transplantation. The CTS Eye Banks in the UK and Europe have used organ culture storage of donor corneas at 34°C for this purpose.3 The use of organ culture for corneal storage as opposed to other short-term storage media (e.g., Optisol in the UK CTS Eye Banks) is generally due to the short supply of corneas available for transplantation in the United Kingdom. The extended storage time allows corneas to be stored for up to 28 days, ensuring reduced wastage due to outdating and that a supply of corneas is always available for emergency procedures. Other benefits of an extended storage time include routine surgical procedures, virological testing, and tissue-typing where necessary. Contaminated corneas are discarded before transplantation as a result of microbiologic screening, and the evaluation of corneal endothelium ensures a high quality of donor tissue for transplantation.

The standard prerequisite of an endothelial cell count greater than 2200 cells/mm2 in all transplantable donor corneas in the CTS Eye Banks ensures a sufficient cell reserve to withstand the stresses of transfer from donor to recipient and will maintain quality of vision for many years after surgery.4 However, this results in rejection of over 30% of organ-cultured donor corneas at the time of endothelial assessment before transplantation due to endothelial deficiencies.5 Previous studies have shown that deterioration of the corneal endothelium occurs with increasing length of organ culture storage time.3–7

Such deterioration of corneal endothelium during organ culture may be a result of cell loss due to apoptosis or necrosis. Apoptosis is a physiological and active mode of cell death that is tightly controlled in multicellular organisms to maintain tissue homeostasis.8 Morphologically apoptotic cells can be identified by condensation and fragmentation of the nucleus. In contrast to necrotic cell death, the plasma membrane of apoptotic cells remains intact, and apoptotic cells are rapidly recognized and phagocytosed without detriment to neighboring viable cells. Biochemical hallmarks of apoptosis are the nonrandom cleavage of DNA into 50-kbp fragments that are (in most cell types) subsequently cleaved to 180-bp integers, which can be readily identified using the TUNEL technique and agarose gel electrophoresis.9 Various studies have also demonstrated the pivotal role of caspases in mammalian cell apoptosis.10

The aim of this investigation was to determine whether deterioration of the human corneal endothelium during organ culture storage resulted from cell loss due to CEC apoptosis.

METHODS
Source of Tissue
Twenty-eight human donor corneas from 20 donors, between 1.5 and 87 years of age, were obtained from the Manchester
Eye Bank. All corneas were received from donors with specific consent for research purposes and those contraindicated for transplantation due to cause of death but without endothelial insufficiency. All eyes were retrieved within 36 hours of cadaver time and were received as whole eyes in moist chamber at 4°C.

Organ Culture Storage

Corneoscleral discs were excised and stored using the organ culture technique according to standard CTS Eye Bank operating procedures. After thorough cleansing of whole eyes (four rinses in sterile 0.9% [wt/vol] NaCl, a 3-minute immersion in 5% polyvinylpyrrolidone iodine, neutralization in 2% sodium thiosulphate, and a final wash in 0.9% [wt/vol] NaCl), corneoscleral discs were excised. Corneoscleral discs were suspended in 80 ml of organ culture medium in a glass DIN bottle, using a 5/0 Mersilk suture (Ethicon, AAH, Bristol, UK) through the scleral rim. Organ culture medium consisted of Eagle’s minimum essential medium (MEM) with Earle’s salts and 25 mM HEPES buffer (GIBCO, Glasgow, UK), containing 2% fetal calf serum, 2 mM glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 0.25 μg/ml amphotericin B. Corneas were stored in a standard incubator at 34°C for varying lengths of time, up to 30 days, with medium remaining unchanged throughout storage.

Endothelial Assessment

After a defined storage time, endothelial cell density and viability were evaluated in an area of 1.54 mm² using phase-contrast light microscopy according to standard CTS Eye Banking protocols. The corneal endothelium was stained for 1 minute in 0.45% trypan blue (Sigma, UK) to identify the number and distribution of dead cells. After a rinse in sterile 0.9% (wt/vol) NaCl, hypotonic 1.8% sucrose was applied to the corneal endothelium for visualization of cell borders. A cell count in the central corneal endothelium was calculated (expressed as the number of endothelial cells per square millimeter) using an eyepiece graticule calibrated to the microscope stage, and the degree of folding was noted. The quality of the endothelium was assessed and graded 1 to 5, according to the evaluation criteria demonstrated in Table 1. Corneoscleral discs were fixed immediately post assessment in 10% neutral-buffered formalin and stored at 4°C.

<table>
<thead>
<tr>
<th>Grade</th>
<th>Assessment</th>
<th>Cell Count/mm²</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Excellent</td>
<td>&gt;2800</td>
<td>No dead cells</td>
</tr>
<tr>
<td>2</td>
<td>Very good</td>
<td>2600–2799</td>
<td>1 or 2 dead cells</td>
</tr>
<tr>
<td>3</td>
<td>Good</td>
<td>2500–2599</td>
<td>Dead cells &gt; 2 &lt; 10</td>
</tr>
<tr>
<td>4</td>
<td>Poor</td>
<td>2000–2299</td>
<td>Moderate folding</td>
</tr>
<tr>
<td>5</td>
<td>Very poor</td>
<td>&lt;2000</td>
<td>Many dead cells</td>
</tr>
</tbody>
</table>

Corneal endothelium was graded from 1 to 5, in order of decreasing quality, as assessed by endothelial cell count, number of trypan blue-positive cells, and corneal folding.

Analysis of Apoptosis

TUNEL Technique. After fixation, in 10% neutral-buffered formalin as described above, each cornea was thoroughly washed in three 10-minute changes of phosphate-buffered saline (PBS). Whole corneas were mounted endothelial side up on glass slides. Endogenous peroxidase was quenched using 2% H₂O₂ in PBS for 15 minutes. Cell membranes were permeabilized by a 20-minute incubation in 20 μg/ml proteinase K, followed by a 20-minute incubation in 0.1% Triton X-100. Apoptotic cells in the corneal endothelium were then identified using the TdT-dUTP terminal nick-end labeling (TUNEL) technique (Oncor cell death in situ detection kit; Appligene Oncor UK, Watford, Herts, UK), a histochemical technique that labels the free ends of the nuclear DNA fragments characteristic of apoptosis.

The corneal endothelium was incubated in TdT in a humidified atmosphere at 37°C for 2 hours, rinsed in three washes of PBS, and incubated in peroxidase-tagged anti-digoxigenin antibody for 1 hour at room temperature. After thorough washing in PBS, diaminobenzidine was applied to the corneal endothelium for color development of TUNEL-positive cells. After immersion in distilled water, four radial cuts were made in the cornea to allow flatmounting of corneas. TdT was excluded from the DNA nick-end labeling reaction, replaced by distilled water, for negative controls.

TUNEL-positive cell nuclei were visualized and analyzed using light microscopy. Apoptosis in each cornea was expressed as a percentage of cells and as the number of labeled cells per unit area, and quantitated in 10 fields of view of 0.0625 mm² in areas of folds and 5 fields of view of 0.25 mm² in the central and the peripheral corneal endothelia. Apoptosis in endothelium of organ-cultured human corneas was assessed in corneas at different periods of storage and from different-aged donors.

Hoechst 33345 Staining. Human corneal endothelium was immersed in 0.2 μg/ml Hoechst 33345 (bis-benzamide; Sigma) in the dark for 10 minutes. After thorough washing in PBS, four radial cuts were made into the cornea to allow flatmounting in antifade mountant Gelvatol (Fisons, UK). Nuclear Hoechst-stained DNA was then observed via UV light using an Olympus IX-70 microscope (Olympus, London, UK).

Immunohistochemical Detection of Caspase-3 Activity. Organ culture stored human corneas were fixed in 4% paraformaldehyde overnight. Each cornea was then rinsed in three washes of PBS, before a 1-hour incubation in 0.3% Triton X-100 in PBS to permeabilize cell membranes. After three washes in PBS, the cornea was incubated overnight in anti-human caspase-3 active antibody (R&D Systems, Abingdon, UK; antibody detects the p20 subunit of caspase-3 but not the inactive precursor), diluted 1 in 2500 in PBS. In control samples the primary antibody was replaced with 5% goat serum. The tissue was then washed in PBS, blocked in 5% normal goat serum for 1 hour and washed in PBS, before a 2-hour incubation in cy3-conjugated affinipure goat anti-rabbit IgG antibody (Jackson ImmunolResearch Laboratories, Luton, Beds, UK) diluted 1:300 in PBS. Four cuts were then made in each cornea to allow flatmounting in antifade mountant Gelvatol (Fisons, UK). Caspase-3-related immunofluorescence was then observed using an Olympus IX-70 microscope.
Statistical Analysis
Spearman's rank correlation was used to test the tendency of values of \( y \) to increase (or decrease) as values of \( x \) increase. Spearman's rank correlation coefficient is defined as \( r_s \). Student’s t-test was used to determine the difference between the number of TUNEL-labeled cells in areas with and those without folds. Values of significance were taken as \( P < 0.05 \).

RESULTS
TUNEL Labeling
Apoptotic cells were identified in the corneal endothelium of human organ cultured corneas: Their number and distribution demonstrated a close correlation with corneal folding and the overall quality of the corneal endothelium. CEC apoptosis appeared to be independent of cause of donor death, death to enucleation time, and death to culture times (Table 2).

Apoptosis was confirmed by the presence of morphologic alterations characteristic of apoptotic cell nuclei (Figs. 1A through 1C). Chromatin condensation and nuclear blebbing in human CECs was demonstrated by Hoechst staining (Fig. 1A) and the TUNEL technique (Figs. 1B, 1C). Caspase-3 activity was also demonstrated by immunolabeling in areas between folds and along folds (Fig. 1D), providing further evidence for CEC apoptosis.

TUNEL labeling of cells was seen throughout the endothelium and along corneal folds (Fig. 1E). The presence of TUNEL-positive apoptotic cells appeared to be age-dependent. TUNEL-positive cells were not observed in corneas from a 1.5-year-old donor (Fig. 1F), and very few were identified in corneas from an 18-year-old donor, Figures 1G(i) and 1G(ii). However,
TUNEL-positive cells were scattered across the corneal endothelium, often at high density, in all donors over 40 years of age as is demonstrated in Figures 1G(i) and 1G(ii). The total number of apoptotic cells increased from 1.5% in donors less than 20 years of age to greater than 15% in donors aged 61 to 80 years. Despite this, a significant correlation between donor age and percentage apoptosis was not found ($P = 0.652$, $r_s = 0.089$). In all corneas analyzed where TUNEL labeling was identified, the spatial distribution of TUNEL-positive cells demonstrated a higher concentration of apoptotic endothelial cells along areas with corneal folds, Figures 1G(ii) and 1H(ii), significant in both central and peripheral corneas ($t = -4.643$, and $t = -4.681$, $P < 0.001$, respectively). This association was demonstrated in Figure 2, where a positive significant correlation between the number of cells in unfolded areas and areas with folds was confirmed using Spearman’s rank correlation, where $r_s = 0.709$ and 0.629, $P < 0.001$, in both central and peripheral areas of endothelium, respectively. The overall number of apoptotic cells appeared to be greater in the peripheral cornea. This is not surprising because there is an overall increase radially in the number of endothelial cells from the center of the cornea outward.

The temporal distribution of TUNEL-labeled endothelial cells demonstrated an insignificant correlation with length of storage time in organ culture (Fig. 3, not significant at $P = 0.882$, Spearman’s rank correlation coefficient, $r_s = 0.029$). Except in one 37-year-old cornea, significant numbers of TUNEL-positive cells (greater than 5% apoptosis) were only apparent in donor corneas stored for more than 14 days.

As expected, the percentage of TUNEL-positive cells demonstrated a significant correlation with endothelial cell count (Fig. 4, Spearman’s rank correlation coefficient, $r_s = -0.543$, $P < 0.005$) and the overall quality of the endothelium (Fig. 5, Spearman’s rank correlation coefficient, $r_s = 0.707$, $P < 0.001$), as determined at the time of endothelial assessment (see Table 1). Percentage apoptosis was least in corneas that had an excellent endothelium and greatest in those assessed as having a poor or very poor endothelium.

**DISCUSSION**

The integrity of donor corneal endothelium after organ culture storage is critical to recipient graft survival. This investigation has clearly demonstrated that CEC loss during organ culture storage occurs via apoptosis. Although apoptosis was apparent throughout the corneal endothelium, the greatest density of apoptotic cells was localized to corneal folds. The degree of apoptosis showed a positive relationship with donor age but only a weak correlation with storage time. The strong correlation between apoptosis, endothelial cell count, and overall assessment quality of the endothelium indicates that apoptosis has a major impact on the 33% discard rate of human corneas stored in organ culture.

CEC apoptosis has been unequivocally characterized by a variety of techniques: the TUNEL technique, histochemical staining to demonstrate morphologic nuclear changes typical of apoptotic cells, and positive immunostaining for active caspase-3. The lack of evidence of cell membrane permeability, as determined by trypan blue exclusion indicates that apoptosis rather than necrosis is the principal form of cell death in the corneal endothelium under organ culture conditions.

Our observation of CEC apoptosis during Eye Bank storage is supported by a number of studies. The most recent by Komuro et al. reported apoptosis in human corneas stored in optisol at 4°C for up to 21 days. Apoptosis occurred at low levels (up to 3%) but then increased significantly after 16 days of storage. Cell culture studies have demonstrated that endothelial cell apoptosis can be mediated by staurosporine, oxidative stress, and Fas/Fas ligand interaction. The latter system is particularly important because the Fas system is known to be present in the cornea and has been shown to be implicated in keratocyte cell death in the anterior stroma during corneal wound healing.
system, growth factors or their absence, oxidative damage, transformation, viral infection, chemotherapeutic agents, and mechanical stress. The trigger for CEC apoptosis in organ culture is unknown, although a variety of factors may contribute and are likely to be dependent on the local environment of the cell. Such factors, including nutrient deprivation, mechanical stress, endotoxins, and/or loss of survival factors, are discussed below in more detail. In vivo essential metabolites are supplied to the cornea via tears (oxygen) and the aqueous humor (glucose). The latter contains proteins that upregulate bcl-2 gene transcription and protect cultured CECs from apoptosis. Submersion of the cornea in a closed organ culture storage system provides a totally artificial environment that limits oxygen supply and results in glucose depletion and lactate accumulation. Komura et al. have reported that 4°C storage of human corneas can result in cell death in all layers of the cornea, being greatest in kerocytes. They also demonstrated that this was predominantly due to apoptosis with some necrosis that may have resulted secondary to apoptosis. This is suggestive of considerable cross talk between the three cell types in the cornea that may promote apoptosis under adverse conditions. Our study did not investigate cell death in the other layers of the cornea because our main concern was the endothelium.

Mechanical induction is likely to play an important role in CEC apoptosis because there was a strong association with corneal folds, which occur as a consequence of stromal swelling. Stromal swelling is known to deform the cornea such that posterior strain is increased, thereby providing a stimulus for mechanical disruption of cell-cell and/or cell-matrix interactions leading to anoikis. Consistent with this is the overall lower level of endothelial cell apoptosis identified in optisol-stored human corneas in which stromal swelling is greatly reduced compared with that in organ culture storage. Mechanical stress has also been shown to modulate myocyte apoptosis as a result of stretch-mediated activation of p53 in vitro, an abnormal myocardial load in heart disease in vivo, whereas vascular endothelial cell death occurs after removal of hemodynamic forces.

Bacterial lipopolysaccharides or endotoxins, identified in “sterile” organ culture medium, have been shown to instigate caspase-mediated cleavage of cell-cell and cell-matrix adherens junctions and are thus potential activators of CEC apoptosis. However, inhibition of endotoxin-mediated endothelial cell loss in porcine corneas can be achieved by increasing the concentration of fetal calf serum in organ culture.

Our findings suggest that prevention of endothelial cell apoptosis either by inhibition of stromal swelling and/or cell death would reduce the discard rate of human corneas, which in turn will help relieve the Europe-wide shortage of donor tissue. Although the cause of corneal swelling in organ culture is unknown, a mechanism by which to regulate stromal water...
intake and/or output would be pertinent to reduce corneal distension, corneal folds, and, thus, CEC apoptosis. Further studies are necessary in this area.

Factors known to inhibit apoptosis in other cells include the growth factors insulin-like growth factor-1, fibroblast growth factor, and platelet-derived growth factor.\textsuperscript{20,28–30} CEC apoptosis itself has been suppressed by an uncharacterized aqueous humor borne factor.\textsuperscript{14,15} Thus, an understanding and identification of anti-apoptotic factors are required to limit deterioration of the endothelium during organ culture storage and, therefore, reduce the discard rate of donor corneas. It is critical that the donor corneal endothelium has sufficient viable cells to guarantee a clear corneal transplant and to survive a postoperative endothelial cell loss of up to 48%.\textsuperscript{31} Successful identification of such survival factors may also be relevant to the potential prevention of postoperative CEC death in vivo.

\begin{table}
\centering
\caption{Details of Donor Corneas}
\begin{tabular}{ccccccc}
\hline
Donor & Age, y & Death-to-Enucleation Time, h & Death-to-Culture Time, h & Storage Time, d & Assessment Grade & Endothelial Cell Density, cells/mm\textsuperscript{2} & % Apoptosis & Cause of Death \\
\hline
1 & 1.5 & 20.20 & 35.20 & 30 & 1 & 3600 & 0 & Other \\
2 & 2.5 & 7.5 & 13.5 & 30 & 4 & 2400 & 7.2 & Cancer \\
3 & 16 & 1.25 & 22.25 & 20 & 1 & 2800 & 0.85 & Hypoxic brain damage \\
4 & 28 & 14.5 & 34.00 & 24 & 3 & 2400 & 4.73 & RFA \\
5 & 28 & 9.08 & 28.35 & 21 & 3 & 2500 & 6.46 & Cancer \\
6 & 32 & 13.25 & 28.92 & 28 & 5 & 1900 & 4.59 & RFA \\
7 & 35 & 18.50 & 45.75 & 21 & 5 & 2400 & 13.53 & Cardiac arrest \\
8 & 37 & 10.42 & 21.42 & 15 & 5 & 2600 & 4.91 & Suicide \\
9 & 44 & 7.75 & 22.75 & 27 & 4 & 2300 & 12.00 & Fibrosing alveolitis \\
10 & 48 & 42.67 & 63.85 & 10 & 2 & 2600 & 0.8 & Cancer, pneumonia \\
11 & 49 & 19.17 & 29.75 & 30 & 4 & 2300 & 0.75 & SAH \\
12 & 63 & 11.42 & 36.67 & 23 & 5 & 1900 & 15.50 & SAH \\
13 & 63 & 11.67 & 34.25 & 29 & 5 & 1900 & 7.9 & Cancer \\
14 & 69 & 35.5 & 41 & 8 & 1 & 3000 & 2.5 & ICH \\
15 & 70 & 14.5 & 24.75 & 29 & 4 & 2500 & 4.44 & Cancer, IHD \\
16 & 71 & 16.25 & 37.5 & 28 & 5 & 1600 & 2.15 & Cardiac arrest \\
17 & 73 & 7.00 & 24.00 & 24 & 4 & 2200 & 3.48 & SAH \\
18 & 81 & 20.67 & 41.85 & 25 & 4 & 2300 & 4.1 & MI \\
19 & 82 & 53.00 & 54.92 & 19 & 3 & 2800 & 2.00 & Cancer \\
20 & 87 & 18.42 & 40.42 & 35 & 3 & 2500 & 1.75 & IHD \\
\hline
\end{tabular}
\end{table}

IHD, ischaemic heart disease; MI, myocardial infarction; RTA, road traffic accident; SAH, subarachnoid haemorrhage; ICH, intracranial haemorrhage.

References


